

Serial No.: 09/274,752
Filed: March 23, 1999

thereof serve to place this application in a condition of adherence to the rules 37 C.F.R. § 1.821-1.825.

Please direct any calls in connection with this application to the undersigned at (415) 781-1989.

Respectfully submitted,
FLEHR HOHBACH TEST
ALBRITTON & HERBERT LLP



Todd A. Lorenz
Reg. No. 39,754

Four Embarcadero Center, Suite 3400
San Francisco, CA 94111-4187
Telephone: (415) 781-1989

Serial No.: 09/274,752
Filed: March 23, 1999

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

The section beginning on page 46, line 7 that begins with the heading "Example 5: Cloning of Human Edg5 cDNA and Design and Preparation of Mammalian Expression Construct:" has been amended as follows:

--Example 5: Cloning of Human Edg5 cDNA and Design and Preparation of Mammalian Expression Construct:

The human ortholog of rat H218/AGR16 was cloned by a combination of RT-PCR and RACE (rapid amplification of cDNA ends) methods. First, a human cDNA fragment was amplified with degenerate primers corresponding to the amino acid sequence LLAIAIER (SEQ ID NO: 6) (5'-ctcctg/cgccatc/tgciatc/tgaga/cg) (SEQ ID NO: 7) in the third transmembrane domain, and LLLLDSTC (SEQ ID NO: 8) (5'-cagc/gta/ca/ga/ca/gtccagc/gaga/gagc/ga) (SEQ ID NO: 9) in the sixth transmembrane domain of rat H218/AGR16. The cDNA template for the PCR reaction (35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min on Stratagene's Robocycler) was reverse-transcribed products of polyA+ RNAs isolated from human neuroblastoma cell line SK-N-MC. A 400 bp product was obtained and sequenced, which has a DNA sequence 80% identical to the corresponding region of the rat H218/AGR16.

The rest of the cDNA sequence was then obtained by 5'- and 3'-RACE using RACE-ready cDNAs derived from human fetal brain (Marathon-ready human fetal brain cDNA, Clontech). The gene-specific primers in 5'- and 3'-RACE were derived from the 400 bp cDNA

Serial No.: 09/274,752
Filed: March 23, 1999

fragment (5'-gcaggacagtggagcaggcctcg (SEQ ID NO: 10) and 5'-ctctctacgccaaggcattatgtct, (SEQ ID NO: 11) respectively). The RACE reaction conditions were 35 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 2 min on a Robocycler. RACE products were cloned into pCR2.0 (Invitrogen) and sequenced.

The sequences of RACE products, presumably located 5' and 3' to the original 400 bp PCR products, were highly-similar to the corresponding regions in rat H218/AGR16. The composite sequence of the RACE and the original 400 bp PCR products encoded a protein with an open reading frame for a 353 amino acid protein that is 92% identical to rat H218/AGR16. To obtain the full length cDNA, two primers corresponding to the immediate upstream and downstream of the coding sequence (5'-tcggatccccaccatggcagcttactcg, (SEQ ID NO: 12) and 5'-atctagaccctcagaccaccgttgcctc (SEQ ID NO: 13), respectively) were used to amplify with Marathon-ready human fetal brain cDNA (95°C for 1 min, 55°C for 1 min, 72°C for 2 min with *pfu* polymerase).

The resulting PCR product was cut with EcoRI and XbaI and cloned into pCDEF3 mammalian expression vector. The sequence of the cDNA confirmed that it is consistent with the composite sequence obtained from RACE and original PCR. Like its rat counterpart H218/AGR16, the human protein belongs to the Edg family of GPCRs, with amino acid sequence 43 - 44% identical to human S1P receptors Edg1 and Edg3, and 33 - 35% identical to LPA receptors Edg2 and Edg4. We concluded that it is the human ortholog of rat S1P receptor H218/AGR16, and therefore named it human Edg5.--

Serial No.: 09/274,752
Filed: March 23, 1999

The section beginning on page 47, line 13 that begins with the heading "Example 6: Tsup-1 Cell Expression of Edg Receptors" has been amended as follows:

--Example 6: Tsup-1 Cell Expression of Edg Receptors

The Tsup-1 line of human CD4+8+3^{low} T lymphoblastoma cells is a useful model for studies of the regulation of human T cell apoptosis induced by different immunologically-relevant stimuli. Goetzl et al., J. Cell Biol. 119:493 (1992). Tsup-1 cells also bear surface receptors for many endogenous mediators, that influence thymocyte and T cell apoptosis, including prostaglandins and neuropeptides. The semiquantitative reverse transcription-polymerase chain reaction method described below was used to assess the relative quantity of mRNA encoding each Edg receptor compared to that for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) in unstimulated Tsup-1 cells.

The methodology employed is more fully described in Goetzl et al., J. Immunol (1999), *supra*. Briefly, total cellular RNA was extracted from suspensions of Tsup-1 cells by the TRlzol method (Gibco-BRL, Grand Island, NY), and a Superscript kit (Gibco-BRL) was used for reverse transcription (RT) synthesis of cDNAs. Polymerase chain reaction (PCR) began with a "hot start" at 94°C for 3 min, *Taq* DNA polymerase was added and amplification was carried out with 35 cycles of 30 s at 94°C, 2 min at 55°C and 1 min at 72°C. Two uCi of [α -³²P] dCTP were added to some sets of reaction mixtures to allow quantification of mRNA encoding each Edg receptor relative to that of the standard G3PDH. Kaltreider et al., Am J. Resp. Cell. Mol. Biol. 16:133 (1996).

Serial No.: 09/274,752
Filed: March 23, 1999

Oligonucleotide primer pairs were: 5'-dCCTGGCCAAGGTCATCCATGAC AAC (SEQ ID NO: 6 14) and 5'-dTGTCAACCAGGAAATGAGCTTGAC (SEQ ID NO: 7 15) for G3PDH; 5'-CTACACAAAAAGCTTGGATCACTCA (SEQ ID NO: 8 16) and 5'-CGACCAAGTCTAGAGCGCTCCGGT (SEQ ID NO: 9 17) for Edg-1 (1100 bp); 5'-dGCTCCACACACGGATGAGCAACC (SEQ ID NO: 10 18) and 5'-GTGGTC ATTGCTGTGAACCTCCAGC (SEQ ID NO: 11 19) for Edg-2 (621 bp); 5'-dCAAAATG AGGCCTTACGACGCCA (SEQ ID NO: 12 20) and 5'-dTCCCATTCTGAAGTGCTG CGTTC (SEQ ID NO: 13 21) for Edg-3 (701 bp); 5'-dAGCTGCACAGCCGCCTGCC CGT (SEQ ID NO: 14 22) and 5'-dTGCTGTGCCATGCCAGACCTTGTC (SEQ ID NO: 15 23) for Edg-4 (775 bp); 5'-CTCTCTACGCCAAGCATTATGTGCT (SEQ ID NO: 16 24) and 5'-ATCTAGACCCTCAGACCACCGTGTGCCCTC (SEQ ID NO: 17 25) for Edg-5 (500 bp); 5'-dAGTCCTCAAATCATCCCACATCTGC (SEQ ID NO: 18 26) and 5'-dAAGTGGCACTCCTGTCTCGTAATC (SEQ ID NO: 19 27) for the type I vasoactive intestinal peptide receptor (VPAC1); and 5'-dTCCCAGCAGGTGCCTG GCCTAC (SEQ ID NO: 20 28) and 5'-dCGAGCCTTTGTACTGTGACTGGTC (SEQ ID NO: 21 29) for VPAC2.

PCR products were resolved by electrophoresis in a 2 g/100 ml agarose gel with ethidium bromide staining. G3PDH, VIPR and Edg R bands were cut from gels and solubilized for beta scintillation counting in 0.5 ml of sodium perchlorate solution at 55°C for 1 h (EluQuick, Schleicher and Schuell, Keene, NH). Initially, the G3PDH cDNA templates in several different-sized portions of each sample were amplified to determine volumes that would

Serial No.: 09/274,752

Filed: March 23, 1999

result in G3PDH bands of equal intensity for each sample. Relative quantities of cDNA encoding each Edg receptor also were calculated by the ratio of radioactivity to that in the corresponding G3PDH band. Kaltreider et al., Am. J. Resp. Cell. Mol. Biol. 16:133 (1996). The following results were obtained, with ratio shown being the ratio of ^{32}P in the VPAC or Edg receptor cDNA band to that in the G3PDH band:--

On page 57, immediately preceding the claims, the previously submitted text entitled "SEQUENCE LISTING" was inserted into the text.